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Functional domain organization of human APOBEC3G

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ABSTRACT

Human APOBEC3 proteins exist in two forms containing either a single cytidine deaminase domain (CDA) or two CDAs. Strikingly, the proteins that are capable of effectively inhibiting the infectivity of Vif-deficient HIV-1 (HIV-1ΔVif), such as APOBEC3G (A3G), contain two CDAs. In contrast, single-domain APOBEC3 proteins such as APOBEC3A (A3A) are weak inhibitors of HIV-1ΔVif, even though A3A is an active cytidine deaminase and a potent inhibitor of retrotransposon mobility. Here, we demonstrate that the ability to bind to Gag and package into HIV-1 virions is entirely contained within the amino-terminal half of A3G. By changing three adjacent amino acids in A3A, to the sequence found in the N-terminal half of A3G, we were able to confer on A3A the ability to be efficiently incorporated into HIV-1 virions and to bind HIV-1 Gag. Nevertheless, this A3A mutant remained a weak inhibitor of HIV-1 infectivity, suggesting that segregation of the Gag-binding/virion incorporation and cytidine deaminase/virus-inhibition activities of APOBEC3 proteins into two tandem CDA regions promotes the efficient inhibition of retrovirus infectivity by APOBEC3 proteins.

Introduction

Members of the APOBEC3 family of cytidine deaminases have been shown to function as innate inhibitors of retrovirus replication and retrotransposon mobility (reviewed by Cullen, 2006). Seven APOBEC3 family members have been identified in humans, designated APOBEC3A (A3A) through APOBEC3H (A3H). These proteins can be further subdivided into ~20 kDa proteins containing a single consensus cytidine deaminase domain (CDA), i.e., A3A, APOBEC3C (A3C) and A3H, and ~40 kDa proteins containing two tandem CDAs, i.e., APOBEC3B (A3B), APOBEC3F (A3F), APOBEC3G (A3G) and APOBEC3D/E. How the duplication of the CDA facilitates the inhibitory activity of these latter four proteins has remained largely unclear.

The antiretroviral potential of APOBEC3 proteins was first recognized in the case of A3G, which is a potent inhibitor of the activity of HIV-1 mutants lacking a functional Vif gene product (HIV-1ΔVif) (Sheehy et al., 2002). Infection of A3G-expressing cells by HIV-1ΔVif mutant viruses results in the specific incorporation of A3G into progeny virions. Upon subsequent infection, A3G interferes with the HIV-1 reverse transcription process, at least in part by extensively editing dC to dU on the nascent proviral DNA minus strand (Harris et al., 2003; Mangeat et al., 2003; Yu et al., 2004; Zhang et al., 2003). The overall effect of this is to severely inhibit the production of functional, integrated HIV-1 proviruses. However, wild-type HIV-1 encodes the Vif protein, which binds A3G directly and induces its degradation by targeting A3G to the proteosome, thus protecting HIV-1 from A3G-mediated inhibition (Conticello et al., 2003; Kao et al., 2003; Marin et al., 2003; Mehle et al., 2004; Sheehy et al., 2003; Yu et al., 2003).

While the basis for selective A3G packaging into HIV-1 virions remains incompletely understood, it is known to be mediated by the nucleocapsid domain of the HIV-1 Gag protein (Alce and Popik, 2004; Cen et al., 2004; Luo et al., 2004; Schäfer et al., 2004; Zennou et al., 2004). Within A3G itself, it has been reported that inactivating mutations of the amino-terminal CDA, which extends from approximately residue 65 to 104 in A3G, reduce A3G virion incorporation, while mutation of the carboxy-terminal CDA has little effect (Bogerd et al., 2007; Navarro et al., 2005; Newman et al., 2005). Others have reported that residues 107 to 162, or residues 105 to 156, both lying outside the core CDA, are critical for HIV-1 virion incorporation of A3G (Cen et al., 2004; Luo et al., 2004), and Huthoff and Malim (2007) recently reported that A3G residues 124 to 127, a highly aromatic amino acid stretch consisting of Tyr-Tyr-Phe-Trp (YYFW), play a particularly important role in A3G virion incorporation.

Analysis of the residues in A3G required for Vif binding has indicated that these also lie in the amino-terminal half of the protein. Specifically, Zhang et al. (2008) have reported that A3G residues 105 to 156 are essential for HIV-1 Vif binding but that A3G degradation, surprisingly, also requires residues 157 to 245. Others have demonstrated that the individual mutation of A3G residues 128–130 can totally block HIV-1 Vif binding (Bogerd et al., 2004; Huthoff and Malim, 2007; Schrofelbauer et al., 2004). While the amino-terminal half of A3G therefore contains all the sequences required for HIV-1 virion incorporation and Vif binding, inhibition of HIV-1 infectivity also requires the carboxy-terminal half of A3G. At

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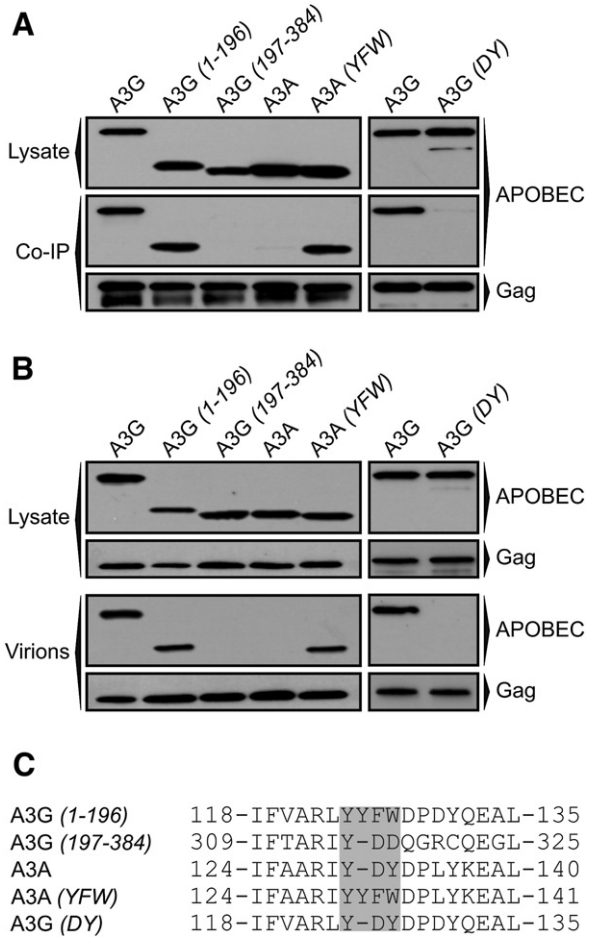

Fig. 1. Binding of APOBEC3 proteins to HIV-1 Gag correlates with virion packaging. (A) The ability of wild-type and mutant forms of A3G and A3A to bind HIV-1 Gag was determined by co-expressing in 293T cells the viral polyprotein with the indicated HA-tagged APOBEC3 proteins, followed by co-immunoprecipitation with a rabbit polyclonal HIV-1 p24 antiserum. Proteins present in the lysate and co-immunoprecipitate (Co-IP) were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by Western blotting with a mouse monoclonal antibody against HA (APOBEC) or HIV-1 p24 (Gag). (B) Full-length wild-type A3G and the A3G(DY) mutant, as well as the indicated single-domain APOBEC3 proteins, were expressed in the context of HIV-1ΔVifΔEnv and virion packaging evaluated. Proteins from cell lysates and purified virions were analyzed by Western blotting as described above. (C) Amino acid alignment of selected regions in the APOBEC3 proteins. The highlighted segments show the presence or absence of the four-amino acid YYFW motif previously implicated in the HIV-1 virion encapsidation of A3G (Huthoff and Malim, 2007).

least in part, this appears to reflect the fact that only the carboxy-terminal CDA is, in fact, enzymatically active as a cytidine deaminase (Hache, Liddament, and Harris, 2005; Iwatani et al., 2006; Miyagi et al., 2007; Navarro et al., 2005; Newman et al., 2005; Schumacher et al., 2008).

While A3G, as well as A3B and A3F, can all block HIV-1 infectivity efficiently, other human APOBEC3 proteins have relatively weak inhibitory activity (Doehle et al., 2005; Wiegand et al., 2004; Zheng et al., 2004). In the case of A3A, recent data demonstrate that inhibitory activity can be induced by fusion of A3A to the amino-terminal half of A3G or to the HIV-1 Vpr protein, both of which induce the recruitment of A3A into HIV-1 virion cores (Aguiar et al., 2008; Goila-Gaur et al., 2007). These observations suggest that A3A, which has a highly enzymatically active CDA (Bogerd et al., 2007), is unable to restrict HIV-1 infectivity only because it is not selectively incorporated into HIV-1 virion particles.

In this manuscript, we confirm and extend these data by analyzing the virion incorporation, HIV-1 Gag binding capacity and virus inhibitory activities of A3G and A3A mutants. In the case of A3G, we show that the amino-terminal half (residues 1–196) of the 384 aa A3G protein is sufficient for HIV-1 virion incorporation and HIV-1 Gag binding, while the carboxy terminal half (residues 197–384) neither binds HIV-1 Gag nor is taken up into virion particles. Neither the A3G (1–196) nor the A3G(197–384) protein was able to inhibit HIV-1ΔVif infectivity when expressed in virus producing cells. The A3G(1–196) protein, but not A3G(199–384), also binds HIV-1 Vif avidly. However, A3G(1–196) is resistant to degradation and is therefore capable of titrating out the Vif protein and thereby facilitating the inhibition of wild-type HIV-1 infectivity induced by full-length A3G. Like A3G(197–384), the wild-type A3A protein is not packaged into HIV-1 virion particles. However, mutation of the analogous residues in A3A to the critical 124-YYFW-127 packaging motif present in A3G resulted in efficient incorporation of A3A into HIV-1 virion particles. Surprisingly, however, this was not reflected in an increased inhibition of virus infectivity.

Results

Sequence requirements for virion incorporation and Gag binding by APOBEC3 proteins

To more fully define the contributions of the amino-terminal and carboxy-terminal halves of the A3G protein, each of which contains a complete CDA, to the inhibition of HIV-1 infectivity, we expressed A3G residues 1–196 and 197–384 as HA-tagged proteins and compared their ability to bind HIV-1 Gag and to package into HIV-1 virions. For this purpose, vectors expressing wild-type A3G, A3G(1–196), or A3G (197–384) were co-transfected into 293T cells with an HIV-1 Gag expression plasmid or a ΔVifΔEnv provirus vector. Virion incorporation was assessed by Western analyses of ΔVifΔEnv HIV-1 virus-like particles collected from the supernatant media. In parallel, HIV-1 Gag binding was assessed by immunoprecipitation of HIV-1 Gag using a polyclonal rabbit p24 capsid antiserum followed by Western analysis of the immunoprecipitate. In both cases, mouse monoclonals specific for HIV-1 Gag or the HA epitope tag were used.

As shown in Fig. 1A, both wild-type A3G and A3G(1–196) bound to HIV-1 Gag efficiently while A3G(197–384) failed to interact. As expected, Gag binding fully predicted the ability to incorporate into the HIV-1 virion particles that were released by the transfected cells (Fig. 1B).

As noted above, A3A is a single CDA domain human APOBEC3 protein that does not effectively inhibit HIV-1 infectivity, even though it is a highly active cytidine deaminase and a potent inhibitor of retrotransposon mobility (Bogerd et al., 2006a, 2006b; Chen et al., 2006; Muckenfuss et al., 2006). As shown in Fig. 1A, A3A is not able to bind HIV-1 Gag effectively and this correlates with undetectable incorporation into HIV-1 virion particles (Fig. 1B). A comparison of the sequences of A3G and A3A showed that the 124-YYFW-127 sequence motif found in the amino terminal region of A3G, which has been reported to be critical for A3G incorporation into HIV-1 virions (Huthoff and Malim, 2007), is lacking in not only the C-terminal region of A3G but also in A3A (Fig. 1C). We therefore asked if mutagenesis of A3A, to substitute the YDY sequence normally present in A3A with this YYFW motif (Fig. 1C), would enhance HIV-1 Gag binding and virion incorporation. As shown in Fig. 1, the resultant A3A(YFW) mutant now bound HIV-1 Gag effectively and was incorporated into HIV-1 virions. Similarly, substitution of the wild-type YYFW motif present in A3G with the YDY sequence found in A3A, in mutant A3G(DY) (Fig 1C), blocked the incorporation of full-length A3G into HIV-1 virions (Fig. 1B) and also prevented binding to HIV-1 Gag (Fig. 1A). These data confirm the importance of the 124-YYFW-127 motif in A3G for HIV-1 Gag binding and virion incorporation and argue that its absence in wild-type human A3A largely explains the poor incorporation of this protein into HIV-1 virion particles.

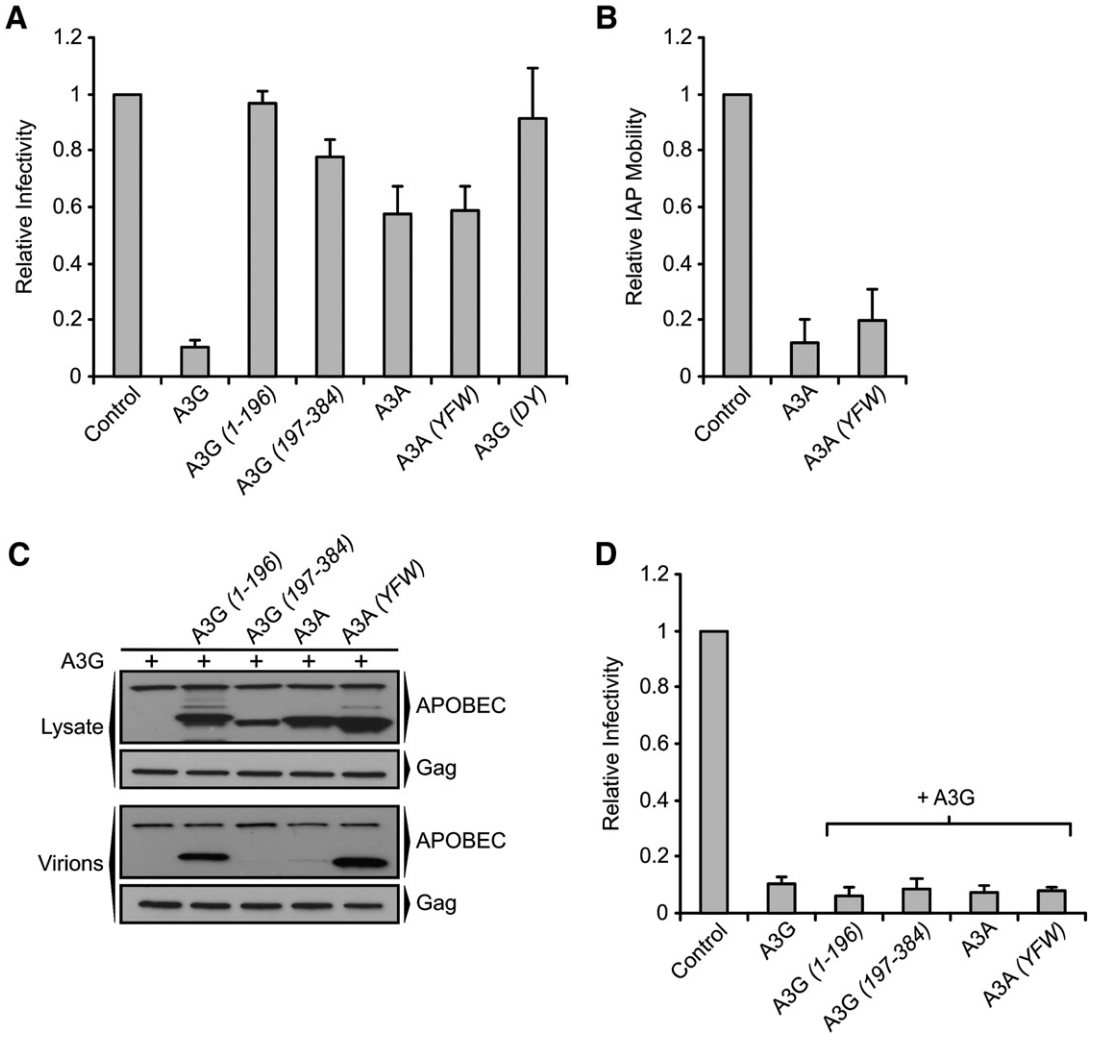

Fig. 2. Packaging-competent single-domain APOBEC3 proteins do not inhibit HIV-1 infectivity. (A) The inhibitory activity of wild-type A3G, the A3G(DY) mutant, and each single-domain APOBEC3 protein against HIV-1 was quantified using the proviral reporter plasmid pNL-Luc-HXBΔVif. 50 ng of the wild-type or full-length A3G expression plasmid, or 200 ng of each single-domain APOBEC3 vector, or of the A3G(DY) mutant expression plasmid, were co-transfected with the proviral construct. Virus-containing media were collected and used to infect 293T cells. The infected cells were lysed and the level of luciferase activity measured. Results were normalized to a transfection lacking an APOBEC3 vector (control). (B) The ability of wild-type A3A and the A3A(YFW) mutant to inhibit the mobility of the IAP LTR retrotransposon was assayed as previously described (Bogerd et al., 2006a). In this assay system, IAP retrotransposition results in resistance to the antibiotic G418 and can be quantitated by counting the number of resistant colonies after co-transfection with an IAP-based indicator construct and drug selection. Data are presented as a fraction of the number of retrotransposition events seen in the absence of any APOBEC3 protein. Average of three experiments with standard deviation indicated. (C) Full-length A3G and the single-domain APOBEC3 proteins were co-expressed in the context of HIV-1ΔVifΔEnv-expressing cells and the amount of A3G encapsidation evaluated. 293T cells were co-transfected with 50 ng phA3G-HA and 200 ng of a single-domain APOBEC3 vector. Proteins from cell lysates and isolated virions were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by Western blotting with a mouse monoclonal antibody against HA (APOBEC) or HIV-1 p24 (Gag). (D) The inhibitory activity of A3G, co-expressed with each single-domain APOBEC3 protein, was examined. In this case, 50 ng of phA3G-HA were co-transfected with 200 ng of each single-domain APOBEC3, along with a reporter provirus. Infection of 293T cells and quantification was carried out as described in A. The results were normalized to a transfection lacking any APOBEC3 vector (control).

Virion incorporation is not sufficient for antiviral activity by A3A

Given recent data showing that A3A can inhibit HIV-1 infectivity when recruited into HIV-1 virion particles by fusion to the amino-terminal half of A3G or to the HIV-1 Vpr protein (Aguiar et al., 2008; Goila-Gaur et al., 2007), we were curious whether the A3A(YFW) mutant would have acquired antiviral activity against HIV-1 due to its enhanced virion incorporation (Fig. 1B). However, we did not see significant antiviral activity from any of the single CDA domain APOBEC3 proteins, including A3A(YFW). Only the wild-type A3G protein could inhibit HIV-1 infectivity effectively, and this inhibition was blocked in the A3G(DY) mutant (Fig. 2A). It seemed possible that the mutation introduced into A3A(YFW), while conferring the ability to bind HIV-1 Gag and package into HIV-1 virion particles (Fig. 1) might also have inhibited the biological activity of A3A. To address this question, we asked if the A3A(YFW) mutant retained its ability to inhibit the mobility of the murine LTR-retrotransposon IAP in co-expressing cells (Bogerd et al., 2006a). As shown in Fig. 2B, both A3A and the A3A(YFW) mutant in fact inhibited IAP retrotransposition by the same ~8 fold. Moreover, A3A and A3A(YFW) also inhibited the mobility of the human non-LTR retrotransposon LINE1 to a similar degree and A3A(YFW) also retained the ability to induce a hypermutation phenotype when expressed in bacteria (data not shown), thus indicating that the A3A(YFW) mutant retained cytidine deaminase activity (Bogerd et al., 2006b). We therefore conclude that the inability of the A3A(YFW) mutant to inhibit HIV-1 infectivity is not due to loss of biological activity.

As the A3G(1–196) and the A3A(YFW) mutants are effectively incorporated into HIV-1 virion particles, yet do not inhibit HIV-1 infectivity, we were curious to see if overexpression of A3G(1–196) or

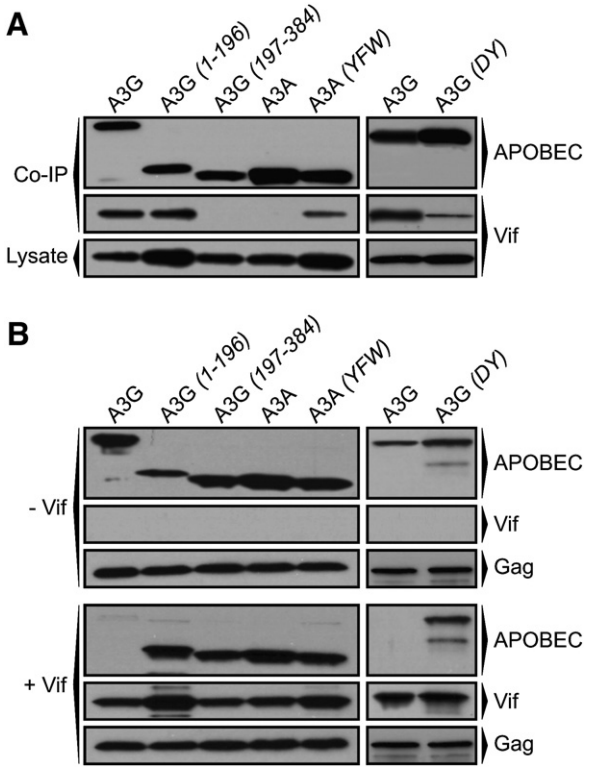

Fig. 3. Binding of single-domain APOBEC3 proteins to HIV-1 Vif does not correlate with Vif-sensitivity. (A) The ability of wild-type or mutant full-length A3G, and of various single-domain APOBEC3 proteins, to bind HIV-1 Vif was determined by co-expressing Vif with the indicated HA-tagged APOBEC3 proteins in 293T cells, followed by immunoprecipitation with a mouse monoclonal anti-HA antibody. Proteins present in the lysate and co-immunoprecipitate (Co-IP) were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by Western blotting with a rabbit polyclonal antibody against HA (APOBEC) or rabbit polyclonal antisera specific for HIV-1 Vif (Vif). (B) Each wild-type or mutant APOBEC3 protein's sensitivity to HIV-1 Vif was compared to that of wild-type A3G by co-transfecting into 293T cells the respective APOBEC3 expression constructs together with empty pcDNA3 (−Vif) or pcDNA-HVif (+Vif) and pNL4-3ΔVifΔEnv. Cell lysates were harvested and protein expression levels analyzed by Western blotting with a rabbit polyclonal antisera specific for the HA epitope (APOBEC) or HIV-1 p24 (Gag). The presence of HIV-1 Vif was probed with the rabbit polyclonal antisera described in panel A.

A3A(YFW) in *trans* would reduce the ability of wild-type A3G to inhibit HIV-1 infectivity by competing for packaging into virion particles. As shown in Fig. 2C, while A3G(1–196) and A3A(YFW) were indeed efficiently incorporated into HIV-1 virions produced in their presence, this did not reduce the incorporation of a constant level of wild-type A3G expressed in the same cells. Moreover, neither the A3G (1–196) protein nor the A3A(YFW) protein, or indeed A3G(197–384) or wild-type A3A, had any obvious effect on the level of inhibition of HIV-1 infectivity induced by wild-type A3G (Fig. 2D). We therefore conclude that the incorporation of A3G into HIV-1 virions is not readily saturated, as also previously reported by Xu et al. (2007).

Vif binding by A3G and A3A mutants

As noted above, the domain in A3G required for Vif binding has been previously mapped to the amino-terminal half of the protein, closely proximal to, but distinct from, the sequences required for HIV-1 Gag binding (Huthoff and Malim, 2007; Zhang et al., 2008). Analysis of the two A3G "half-proteins" confirmed these earlier reports, as both A3G and A3G(1–196) were found to bind HIV-1 Vif effectively, while the A3G(197–384) protein failed to interact (Fig. 3A). Surprisingly, while wild-type A3A also failed to bind HIV-1 Vif, the A3A(YFW) mutant had acquired the capacity to interact with Vif, thus suggesting

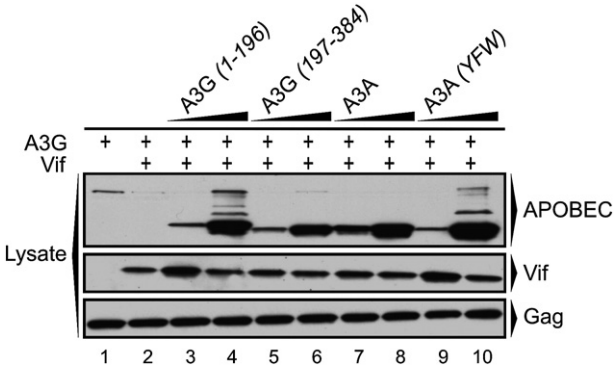

Fig. 4. Vif-binding single-domain APOBEC3 proteins can rescue A3G from Vif-mediated degradation. 293T cells were co-transfected with 50 ng phA3G-HA, 50 ng pcDNA-HVif, and 200 ng (lanes 3, 5, 7, 9) or 500 ng (lanes 4, 6, 8, 10) of a vector encoding a single-domain APOBEC3 protein. The proviral clone pNL4-3ΔVifΔEnv was also co-transfected. Cell lysates were harvested and protein expression levels analyzed by Western blotting with mouse monoclonal antibodies specific for the HA epitope (APOBEC) or HIV-1 p24 (Gag). The presence of HIV-1 Vif was probed with a rabbit polyclonal antisera.

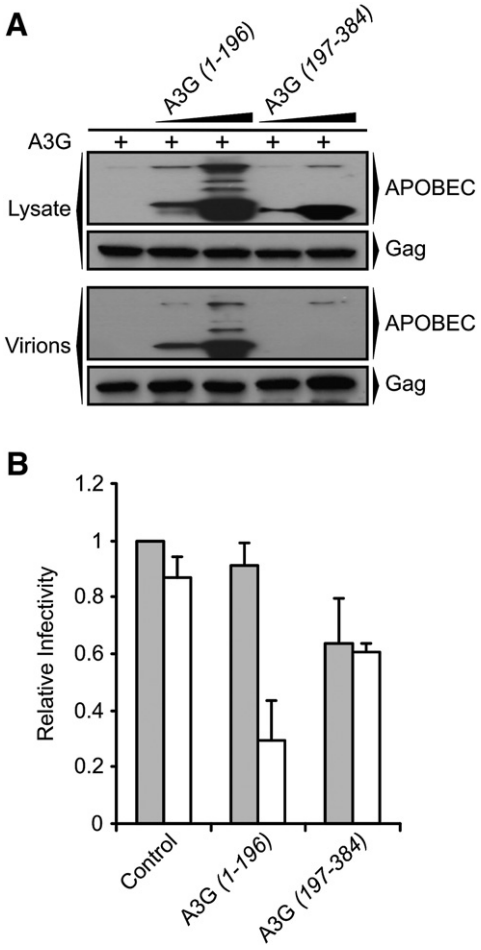

Fig. 5. Rescue of wild-type A3G inhibition of HIV-1 by inhibition of Vif function. (A) Expression plasmids were co-transfected into 293T cells as described in Fig. 4, with two exceptions: the wild-type proviral reporter construct pNL-Luc-HXB was used in place of pNL4-3ΔVifΔEnv and pcDNA-HVif was omitted. The experiment resulted in a similar rescue of full-length A3G expression by A3G(1–196) in the presence of Vif and thereby also enhanced A3G virion incorporation. (B) The effect of the enhanced A3G expression induced by A3G(1–196) on wild-type HIV-1 virion infectivity is represented by the white columns. These data show virus infectivity in the presence of only the single-domain APOBEC3 proteins, i.e., in the absence of A3G (gray columns), or in the presence of both full-length A3G and the truncated A3G protein (white columns). The control samples display wild-type HIV-1 infectivity in the absence of any APOBEC protein (gray column) or in the presence (white column) of full-length A3G only.

that the Gag and Vif binding domains in the A3G must extensively overlap. However, while the A3G(DY) mutant had lost the ability to interact with HIV-1 Gag (Fig. 1A), it retained a reduced but readily detectable ability to interact with the HIV-1 Vif protein (Fig. 3A). Analysis of the ability of HIV-1 Vif to induce degradation of these APOBEC3 proteins revealed that wild-type A3G was effectively degraded while both A3G(1–196) and A3A(YFW) were unaffected (Fig. 3B). These data, while in some ways surprising, nevertheless confirm the previous observation (Zhang et al., 2008) that the Vif binding site on A3G can be mutationally segregated from the sequences required for A3G proteosomal degradation. Moreover, Vif also failed to induce significant degradation of the A3G(DY) mutant (Fig. 3B), despite its ability to bind A3G(DY) in co-expressing cells (Fig. 3A). This result is similar to data reported recently by Marin et al. (2008), who reported that HIV-1 Vif can bind to two other members of the APOBEC3 protein family, A3B and A3C, without inducing a significant level of degradation *in vivo*.

Inhibition of HIV-1 Vif function

If A3G(1–196) and A3A(YFW) can bind Vif while not being degraded, then these proteins should have the potential to act as competitive inhibitors of Vif function by sequestering Vif into an inactive complex. Indeed, as shown in Fig. 4, expression in *trans* of either A3G(1–196) or A3A(YFW) rescued the expression of wild-type A3G in Vif-expressing cells. In contrast, neither A3G(197–384) nor wild-type A3A, neither of which is able to bind to Vif (Fig. 3A), was able to exert this protective effect (Fig. 4).

If A3G(1–196) can protect wild-type A3G from degradation in Vif-expressing cells, then it should also rescue the incorporation of A3G into HIV-1 virions and thereby indirectly reduce the infectivity of those virions. To test this hypothesis, we co-transfected 293T cells with a wild-type A3G expression vector, a truncated A3G mutant expression vector and an expression plasmid encoding a Vif+ HIV-1 provirus bearing the luciferase indicator gene in place of Nef. As shown in Fig. 5A, wild-type A3G expression was again enhanced by overexpression of the A3G(1–196) but not the A3G(197–384) protein. More importantly, A3G(1–196) significantly enhanced the ability of wild-type A3G to inhibit HIV-1 infectivity, while the A3G(197–384) protein exerted no detectable effect (Fig. 5B). Therefore, A3G(1–196) can indeed at least modestly boost wild-type A3G stability and function when expressed in *trans* in HIV-1 Vif expressing cells.

Discussion

Although A3A is a highly active cytidine deaminase and a potent inhibitor of several different retrotransposons (Bogerd et al., 2006a; Bogerd et al., 2006b; Chen et al., 2006; Muckenfuss et al., 2006), it nevertheless fails to appreciably inhibit HIV-1 infectivity (Fig. 2A). A3A also fails to interact with HIV-1 Gag in co-expressing cells and is not selectively packaged into HIV-1 virion particles (Fig. 1), although it does package into virions non-selectively when overexpressed at levels that also lead to the incorporation of irrelevant cytoplasmic proteins, such as β-arrestin (data not shown). As fusion of A3A to the amino-terminal half of A3G, or to the HIV-1 Vpr protein, induces both the virion incorporation of A3A and inhibition of HIV-1 infectivity (Aguiar et al., 2008; Goila-Gaur et al., 2007), it appeared that the inactivity of wild-type A3A could be readily explained as being due simply to its inability to be selectively packaged by HIV-1 virions.

In this report, we show that HIV-1 Gag binding by A3A, and HIV-1 virion incorporation, can be induced by mutation of A3A to induce a "YYFW" motif that has been previously reported to be critical for HIV-1 virion incorporation of A3G (Huthoff and Malim, 2007). These data serve to confirm the critical importance of this aromatic amino acid sequence in HIV-1 virion packaging (Fig. 1B) and extend these earlier data by demonstrating that the integrity of the "YYFW" motif in A3G is also critical for HIV-1 Gag binding (Fig. 1A). These observations also allowed us to test the hypothesis that non-fused A3A would be an effective inhibitor of HIV-1 infectivity if it was taken up into HIV-1 virion particles. In fact, however, the A3A(YFW) mutant is not an effective inhibitor of HIV-1 infectivity (Fig. 2A), even though it retains full inhibitory activity against retrotransposons such as IAP (Fig. 2B).

The observation that the 124-YYFW-127 motif in A3G is critical for binding to HIV-1 Gag implies either that this highly hydrophobic sequence is located on the surface of the A3G protein or that mutation of this sequence, by substitution of amino acids found at this same location in A3A, results in the misfolding of the A3G protein. This latter hypothesis seems unlikely, given that substitution of the YYFW motif into A3A, to give the A3A(YFW) mutant, actually confers both HIV-1 Gag binding and virion packaging on the otherwise excluded A3A protein (Fig. 1). Moreover, modelling of the structure of A3G, using the reported APOBEC2 crystal structure as a starting point, as well as the recently reported crystal structure of residues 198 to 384 of A3G, both predict that these aromatic amino acid residues are expressed at the surface of the A3G protein, where they can directly participate in protein–protein interactions (Chen et al., 2008; Zhang et al., 2007).

The finding that A3A, a single CDA-domain APOBEC3 protein, is not an effective inhibitor when packaged into HIV-1 virion particles suggests that the duplication of the CDA domain seen in human A3G, A3F and A3B, all of which are effective inhibitors of a range of retroviruses (Cullen, 2006), may represent an important step in their evolution. Specifically, the finding that A3A is not an effective inhibitor of HIV-1 infectivity when engineered to enter HIV-1 virions by binding to Gag (Figs. 1 and 2), yet is an effective inhibitor when recruited into HIV-1 virions by fusion to either the amino-terminal half of A3G (Goila-Gaur et al., 2007) or the viral Vpr protein (Aguiar et al., 2008), suggests that a single CDA cannot effectively mediate both retroviral packaging and inhibition of HIV-1 infectivity. In other words, effective inhibition of retroviral infectivity requires segregation of these two activities, i.e., Gag binding and virion packaging on the one hand and inhibition of infectivity and cytidine deaminase function on the other hand, into two tandem CDA domains present in a single protein. According to this hypothesis, the single CDA domain A3A protein can only function as an effective inhibitor of HIV-1 infectivity when it is fused to a separate domain that confers packaging, i.e., either the amino-terminal half of A3G or HIV-1 Vpr (Aguiar et al., 2008; Goila-Gaur et al., 2007).

The data presented in this manuscript confirm and extend previous work suggesting that the amino-terminal half of A3G, i.e., residues 1–196, indeed contains all the sequences required for HIV-1 Gag binding and HIV-1 virion incorporation (Cen et al., 2004; Luo et al., 2004; Miyagi et al., 2007; Navarro et al., 2005; Schumacher et al., 2008) (Fig. 1) yet is not capable of inhibiting HIV-1 infectivity (Fig. 2A). Interestingly, overexpression of A3G(1–196) or A3A(YFW), both of which package effectively, failed to reduce the packaging of wild-type A3G expressed in the same cell (Fig. 2C) or inhibition of HIV-1 infectivity by wild-type A3G (Fig. 2D), thus confirming a previous report suggesting that A3G incorporation into HIV-1 virions is not readily saturated (Xu et al., 2007).

In addition to binding Gag, the A3G(1–196) and A3A(YFW) mutants also bound HIV-1 Vif effectively, although neither protein–unlike wild-type A3G–was found to be destabilized by Vif (Fig. 3). These results suggest that the sequences in A3G that mediate binding to HIV-1 Gag and HIV-1 Vif likely overlap extensively, as insertion of the YFW motif previously defined by Huthoff and Malim (2007) as critical for A3G packaging into HIV-1 virions conferred the ability to bind both HIV-1 Gag and HIV-1 Vif (Figs. 1 and 3). Of interest, it has previously been argued that HIV-1 Vif can block A3G virion incorporation without inducing its degradation, a result that would be consistent with the idea that HIV-1 Gag and HIV-1 Vif compete for binding to overlapping sites on the A3G protein (Opi et al., 2007). On the other hand, the A3G(DY) mutant, which had entirely lost the

ability to bind to HIV-1 Gag (Fig. 1A) partially retained its ability to bind the HIV-1 Vif protein (Fig. 3A), thus demonstrating that the HIV-1 Gag and HIV-1 Vif binding motifs in A3G must be at least partly distinct.

Because A3G(1–196) binds Vif yet is not degraded (Fig. 3), we asked if A3G (1–196) would be able to facilitate the inhibition of wild-type HIV-1 by wild-type A3G by sequestering Vif into an inactive complex. In fact, both A3G(1–196) and A3A(YFW) proved able to inhibit the degradation of wild-type A3G by Vif in co-expressing cells (Figs. 4 and 5A), and A3G (1–196) also reduced the infectivity of wild-type HIV-1 virions produced in the presence, but not the absence, of full-length A3G (Fig. 5B). In contrast, A3G(197–384) had no effect in this assay. These data contrast with a recent report (Zhang et al., 2008) which argued that either residues 1–156 or residues 157–384 of A3G, when expressed in cells, can dominantly inhibit the Vif-mediated degradation of wild-type A3G. The reasons for the difference between our data and this earlier report are not clear, but could reflect the expression of different segments of A3G.

In conclusion, our data further confirm the proposal (Navarro et al., 2005) that the amino-terminal and carboxy-terminal CDAs of A3G fulfil distinct roles during inhibition of retroviral infectivity and suggest that the functional separation of the virion packaging and enzymatic activities of A3G into two tandem CDA domains facilitates the efficient inhibition of retroviral infectivity.

Materials and methods

Cell culture and reagents

293T cells were cultured in DMEM containing 10% FBS. All transfections were performed using the calcium phosphate method in six-well plates. Protein A and rProtein G agarose were purchased from Invitrogen. Purified mouse monoclonal and rabbit polyclonal antibodies against hemaglutinin (HA) were purchased from Covance. The mouse monoclonal antibody (Cat.# 3537) (Chesebro et al., 1992) and rabbit polyclonal antisera (Cat.# 4250) specific for capsid (p24) of the HIV-1 Gag polyprotein, as well as the rabbit polyclonal antisera against HIV-1 Vif (Cat.#2221) (Goncalves et al., 2002), were obtained through the AIDS Research and Reference Reagent Program. Reporter virus luciferase activity was quantified using the Luciferase Assay System from Promega and light emission measured using a Turner TD-20/20 luminometer.

Molecular clones

The HIV-1 proviral expression plasmids pNL-Luc-HXB, pNL-Luc-HXBΔVif and pNL4-3ΔVifΔEnv have been previously described (Bogerd et al., 2004), as have the pcDNA3-based expression plasmids phA3A-HA and phA3G-HA (Wiegand et al., 2004). The plasmid psynGag, encoding a codon optimized HIV-1 Gag protein, has also been described (Doehle, Schafer, and Cullen, 2005). HIV-1 Vif was expressed from the partially codon-optimized vector pcDNA-HVif, which was obtained through the AIDS Research and Reference Reagent Program (Cat. # 10077) (Nguyen et al., 2004). pHIT/G was used to pseudotype HIV-1 infection. Vectors expressing either amino acids 1–196 (phA3G 1–196) or 197–384 (phA3G 197–384) of A3G, each containing a C-terminal triple HA epitope tag, were generated from phA3G-HA. Briefly, the desired coding regions were amplified by PCR and subcloned into phA3G-HA, which had been digested with Asp718/EcoRI to remove the A3G coding region, leaving the HA tag intact. The vector expressing the A3A(YFW) mutant, where amino acids D131 and Y132 are replaced with the amino acid sequence YFW, was constructed from phA3A-HA by recombinant PCR. The A3G(DY) mutant, which contains the opposite mutation, was also generated by recombinant PCR. The integrity of all the expression vectors used in this analysis was confirmed by DNA sequencing.

Co-immunoprecipitations

For co-immunoprecipitation of HIV-1 Gag and APOBEC3 proteins, 293T cells were co-transfected with 500 ng of a plasmid expressing a wild-type or mutant APOBEC3 protein and 100 ng of the psynGag vector. At 40 h post-transfection, the cells were lysed with 50 mM Tris, pH 7.4/150 mM NaCl/0.5% NP-40 and the lysate clarified by brief centrifugation. A portion of the clarified lysate was removed for subsequent analysis while the remainder was added to 30 μl of equilibrated Protein A agarose. Two μl of HIV-1 p24 rabbit polyclonal antisera were added to the matrix and the suspension mixed for 3 h at 4 °C. The matrix was washed extensively then suspended in loading buffer. Equal aliquots of the total and bound fractions were subjected to denaturing gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was probed with mouse mAb specific for either the HA epitope or HIV-1 p24 of the Gag polyprotein.

The procedure outlined above was also used for co-immunoprecipitation of APOBEC3 proteins and HIV-1 Vif, with the following exceptions: Expression plasmids for the APOBEC3 proteins and pcDNA-HVif were co-transfected in equal 500 ng amounts. Clarified lysate was added to rProtein G agarose, and the APOBEC3 and Gag proteins were co-immunoprecipitated with a mouse mAb specific for the HA epitope. Following electrophoresis and transfer, the nitrocellulose membrane was probed with rabbit antisera specific for the HA epitope or for the HIV-1 Vif protein.

Viral packaging of APOBEC3 proteins

To examine expression and virion packaging of the APOBEC3 proteins in the context of virus production, 293T cells were co-transfected with 1500 ng pNL4-3ΔVifΔEnv, 100 ng of an APOBEC3 vector, and 500 ng of pcDNA3 filler. In the case of more complex experiments where A3G is expressed concomitantly with one of the single-domain APOBEC3 proteins, cells were co-transfected with 1500 ng of the indicated proviral vector, 50 ng phA3G-HA, and 200 or 500 ng of a single-domain APOBEC3 plasmid. The total amount of DNA transfected was kept constant using the parental pcDNA3 as filler. Approximately 40 h post-transfection, 1.2 ml of virus containing media were collected and passed through a 0.45 μm filter onto 300 μl of a 20% (wt/vol) sucrose cushion in a microcentrifuge tube. Virus was pelleted by centrifugation at 21,000 rcm for 2 h at 4 °C. The media were decanted and the pelleted virus was resuspended in loading buffer. Relative amounts of lysate and virion incorporated APOBEC3 and Gag proteins were analyzed by Western blotting with a mouse mAb for HA or HIV-1 Gag polyprotein.

Virus infection and luciferase assay

293T cells were co-transfected with 1500 ng pNL-Luc-HXB or pNL-Luc-HXBΔVif, indicated amounts of phA3G-HA, 50 ng pHIT/G, and 200 or 500 ng of a single-domain APOBEC3 plasmid. The total amount of DNA transfected was kept constant using the parental pcDNA3 as filler. Approximately 40 h post-transfection, the supernatant media were collected and filtered as described above, and used to infect 293T cells. The virus-producing cells were lysed and probed for expression of the relevant proteins. Infected cells were assayed 24 h later for luciferase activity.

Vif-sensitivity of APOBEC3 proteins

293T cells were co-transfected with 1500 ng pNL4-3ΔVifΔEnv, 200 ng of an APOBEC3 vector, and 500 ng pcDNA-HVif or pcDNA3 parental vector. Cells were harvested 40 h post-transfection and relative protein levels were assayed by Western blot, as described above.

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